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Candidate Housekeeping Genes Require Evaluation before their Selection for Studies of Human Epidermal Keratinocytes

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TO THE EDITOR

The human epidermis is a stratified epithelium organized into basal proliferative and suprabasal postmitotic keratinocytes. A complex differentiation program defines characteristic phenotypes in these cells. Identification of the epidermal differentiation program and elucidation of regulatory intracellular and environmental processes has allowed understanding of the regulation and deregulation of epidermal homeostasis in either, the normal situation, and in pathologies.

In serum-free autocrine culture conditions (Cook *et al.*, 1991), we showed previously that confluence of cultured keratinocytes corresponds to a strong commitment toward epidermal differentiation (Poumay and Pittelkow, 1995). Quantification of mRNA encoding epidermal markers of differentiation has been the most frequently used procedure to characterize the precise phenotype of keratinocytes. We used this approach for studying the role of cell density (Poumay and Pittelkow, 1995; Poumay *et al.*, 1999a), prolactin (Poumay *et al.*, 1999b), exogenous growth factors (De Potter *et al.*, 2001), or cholesterol depletion (Jans *et al.*, 2004) on the phenotype of keratinocytes grown in autocrine culture conditions (Cook *et al.*, 1991).

Today, several procedures are available to identify and quantify mRNA: northern blotting, RNase protection assay, microarray analysis, and quantitative RT-PCR (qRT-PCR). Each procedure quantifies the expression of a gene by measuring a value that is calculated relatively to the measurement performed on the same sample for the expression of a housekeeping gene (HKG), so called for its stable expression independently of any particular cell phenotype. Candidate HKGs, which have been already chosen for studies of keratinocytes, include glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) (Yamazaki *et al.*, 2004), cyclophilin (*1B15*) (De Potter *et al.*, 2001), ribosomal protein large P0 (*RPLP0* or 36B4) (Poumay *et al.*, 1999b), actin (Hanley *et al.*, 2000), tubulin (Zarach *et al.*, 2004). In many studies of keratinocyte differentiation, the expression of these genes seemed to be almost constant when studied by northern blotting. Real-time qRT-PCR is becoming the more generalized tool for gene expression studies as this technology allows quick analysis of a larger number of genes, as well as very precise measurements. Thus, a particular attention must be paid on data normalization, especially regarding the choice of genes that remain stably expressed in each studied circumstances (Ginzinger, 2002).

For studies of epidermal differentiation in the autocrine culture model of keratinocytes, we analyzed the stability of gene expression for candidate HKGs using geNorm analysis program version 3.5 (Vandesompele *et al.*, 2002). The geNorm algorithm defines the internal control gene-stability measurement *M* as the average paired variation of a particular gene with all other candidate genes. Genes with the lowest *M*-values exhibit the most stable expression (Vandesompele *et al.*, 2002). In the present study, the expression of five commonly used candidate HKGs: *GAPDH*, α-tubulin1B (*TUBA1B*), *RPLP0*, TATA-binding protein (*TBP*), ribosomal protein large 13A (*RPL13A*) and keratin 14 (*KRT14*), one specifically epidermal gene presenting a stable expression in the culture model (Poumay *et al.*, 1999a), was measured in 16 samples (four different culture densities of keratinocytes isolated from four different normal donors). As advised (Vandesompele *et al.*, 2002), 18S or 28S ribosomal RNA were not used because of the possible imbalance between ribosomal and messenger RNA fractions. In each culture analyzed at the second passage, total RNA was extracted 48 hours after medium renewal, 2 days before confluence (C −2d), at confluence (C), or both 2 and 4 days after confluence (C + 2d and C + 4d). To avoid the use of putatively co-regulated HKGs during the geNorm analysis, the stabilities of *RPLP0* (*36B4*) (Figure 1a, open circle) and *RPL13A* (Figure 1a, dark rhombus), in comparison with the other candidates, were measured independently. Each average expression stability value (*M*) (Figure 1a) was calculated following the sequential elimination of the least stable HKG in the culture conditions analyzed in this study. In consequence, the slope of the line between two *M*-values was indicative of the difference in expression stability between one gene and the remaining more stable genes listed on the right side of the graph. This analysis revealed that the cutoff *M*-value of *TBP*, *RPL13A*, and *KRT14* was below 0.3, thus their expression can be considered stable across the samples. *RPLP0* exhibits an M-value under 0.5, considered acceptable for valid HKG (Allen *et al.*, 2008), but less stable than *RPL13A*.

Abbreviations: GAPDH, glyceraldehyde-3-phosphate dehydrogenase; HKG, housekeeping gene; RPLP0, ribosomal protein large P0

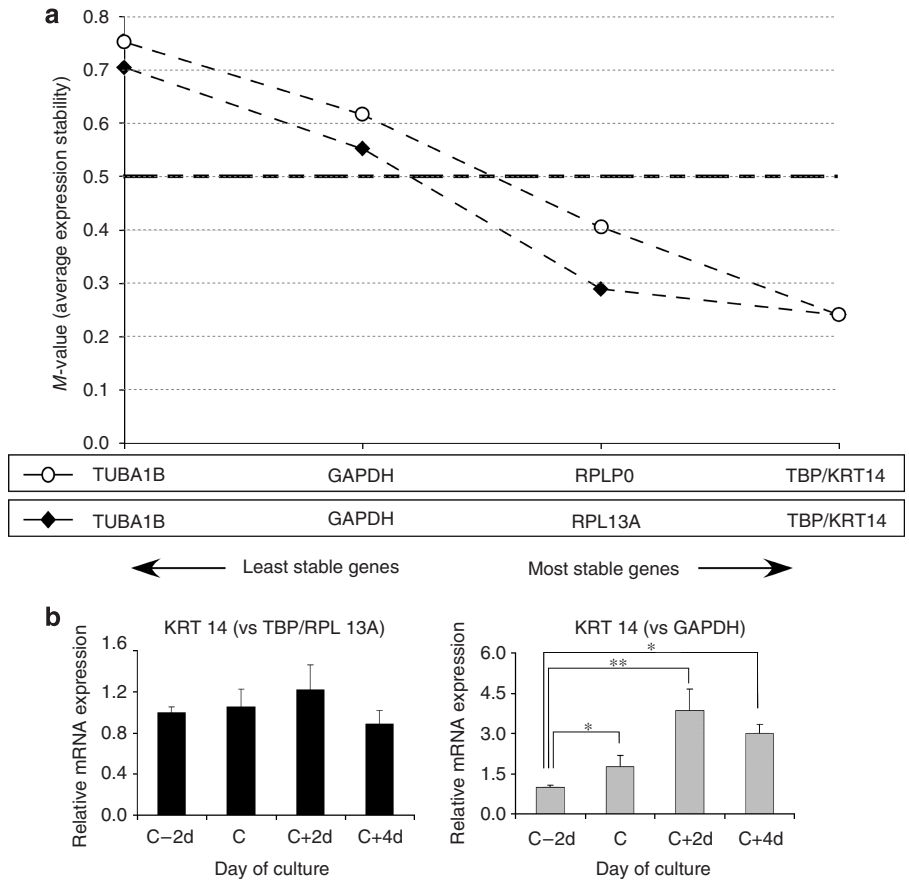

Figure 1. Expression stability of candidate housekeeping genes in human cultured keratinocytes. (**a**) Average expression stability values (*M*) of remaining control genes during stepwise exclusion of the least stable candidate housekeeping gene (Vandesompele *et al.*, 2002) in keratinocytes at four different times of culture isolated from four different normal donors. *M*-values under 0.5 (horizontal dotted line) are considered acceptable for valid HKG (Allen *et al.*, 2008). (**b**) Relative *KRT14* mRNA expression level at four different times of culture of keratinocytes isolated from four different normal donors was normalized either with the geometric mean of *RPL13A* and *TBP* expression levels or with *GAPDH* expression level. Total RNA was extracted, reverse transcription was performed and cDNA was analyzed in duplicate by real-time PCR. Statistical analysis was performed with the paired Tukey–Kramer multiple comparisons test (one-way ANOVA). Data are shown as means ± SEM ($n = 4$) (*$P < 0.05$; **$P < 0.01$). C−2d, 2 days before culture confluence; C, confluence; C + 2d, 2 days after confluence; C + 4d, 4 days after confluence.

Although *RPLP0* remains an excellent HKG candidate, this observation added to the fact that *RPLP0* mRNA may present a modified 5′-UTR sequence that favors the choice of *RPL13A* comparatively with *RPLP0*. In the case of *RPLP0*, as well as for other HKG candidates like hydroxymethylbilane synthase (porphobilinogen deaminase) (Vandesompele *et al.*, 2002; Allen *et al.*, 2008) not evaluated in this study, the expression stability of the different isoforms of these genes should be first established. Regarding *KRT14*, although this gene appears with *TBP* as most stable HKG in these phenotypical conditions, its selection must be avoided as it is known to vary in specific conditions like cholesterol depletion (Jans *et al.*, 2004) or 12-*O*-tetradecanoyl-phorbol-13-acetate treatment (Smits *et al.*, 2000). In contrast with *TBP*, *RPLP0*, *RPL13A*, and *KRT14* genes, the expression of *GAPDH* and α-tubulin (*TUBA1B*) was relatively unstable, as we found *M*-values higher than 0.5 (Figure 1a). These results were confirmed by the NormFinder algorithm (data not shown) (Andersen *et al.*, 2004). Of course, the choice of HKG evaluated in the present study was limited to these few genes already studied in keratinocytes, but is absolutely not exclusive of other potential candidates.

As the geNorm analysis reveals great stability of *KRT14* expression and to illustrate typical error of interpretation due to the choice of inappropriate HKG, the relative mRNA expression of *KRT14* was determined relative to different HKGs. As advised (Vandesompele *et al.*, 2002), the geometric mean of *RPL13A* and *TBP* expression levels was used because these two HKGs present best stabilities (Figure 1a) and indeed no significant variation in *KRT14* expression was observed (Figure 1b). Conversely, the determination relative to *GAPDH* expression, frequently used in many studies, showed significant variations of *KRT14* expression (Figure 1b) unexpected in view of northern data (Poumay *et al.*, 1999a). Accordingly, the relative mRNA expression of *GAPDH* and *TUBA1B*, normalized with the geometric mean of *RPL13A* and *TBP* expression levels, decreased significantly dependently of time of culture (Figure 2). This decrease

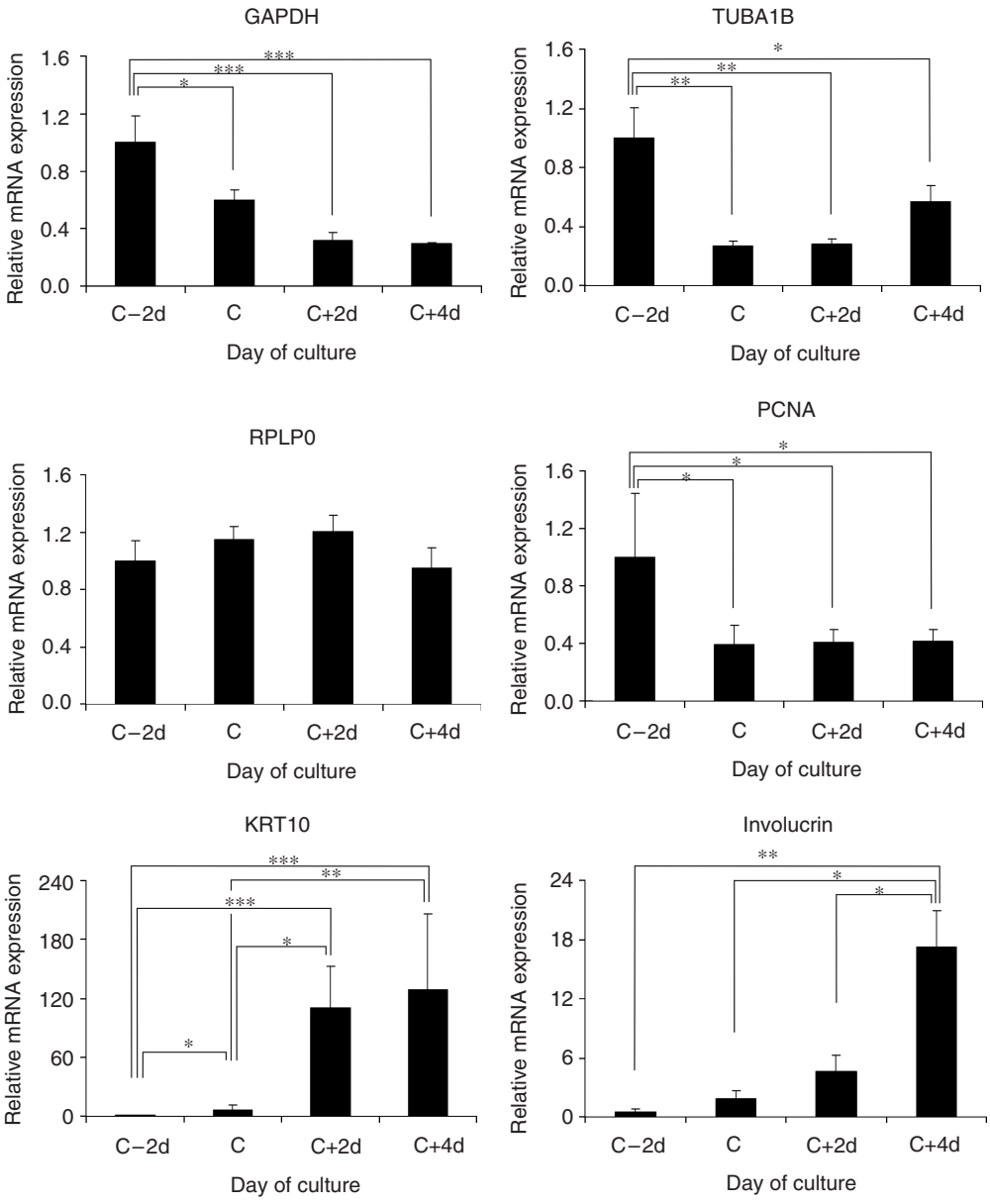

Figure 2. Relative mRNA expression normalized to *RPL13A* and TBP. The mRNA expression determined in samples obtained from four different normal donors at four different times of keratinocyte culture was normalized with the geometric mean of *RPL13A* and *TBP* expression levels. The time of culture of keratinocytes (C−2d, 2 days before culture confluence; C, confluence; C + 2d, 2 days after confluence; C + 4d, 4 days after confluence) downregulates relative expression of glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), *TUBA1B*, and *PCNA* but not *RPLP0*, whereas the relative mRNA expression of involucrin and KRT10 is strongly upregulated. For each sample, total RNA was extracted, reverse transcription was performed and cDNA was analyzed in duplicate by real-time PCR. Statistical analysis was performed with the paired Tukey–Kramer multiple comparisons test (one-way ANOVA). Data are shown as means ± SEM ($n = 4$) (*$P<0.05$; **$P<0.01$; ***$P<0.001$).

was correlated by multiple regression with significant decrease of cell proliferation marker proliferating cell nuclear antigen (*PCNA*) expression level ($P = 0.004$, coefficient = 0.67 and $P = 0.015$, coefficient = 0.6, respectively), conversely to *RPLP0* that remained stable in same conditions. In addition, the expression of differentiation markers was analyzed to confirm the differentiation process in this model (Poumay *et al.*, 1999a): keratin 10 (*KRT10*) and involucrin expression increase very significantly dependently of days of culture (Figure 2). Sequences of primers used in this study are given in Table 1.

Overall findings of this study support the choice of *TBP*, *RPL13A*, and *RPLP0* as HKGs to study differentiation of keratinocytes by real-time qRT-PCR, instead of *GAPDH* or α-tubulin. Our results show that HKGs used in studies of gene expression in keratinocytes must be evaluated dependently of precise experimental conditions before analysis of expression of other genes.

CONFLICT OF INTEREST

The authors state no conflict of interest.

Table 1. Sequences of primers

Gene symbol	Forward primer	Reverse primer
GAPDH	ACCCACTCCTCCACCTTTGAC	GTCCACCACCCTGTTGCTGTA
KRT14	CGATGGCAAGGTGGTGTC	GGGTGAAGCAGGGTCCAG
RPLP0	ATCAACGGGTACAAACGAGTC	CAGATGGATCAGCCAAGAAGG
RPL13A	CTCAAGGTCGTGCGTCTGAA	TGGCTGTCACTGCCTGGTACT
TBP	TCAAACCCAGAATTGTTCTCCTTAT	CCTGAATCCCTTTAGAATAGGGTAGA
TUBA1B	CCCGAGGGCACTACACCAT	CAGGGAGGTGAACCCAGAAC
Involucrin	TGAAACAGCCAACTCCAC	CTCATCCAGCACCCTACG
KRT10	AATCAGATTCTCAACCTAACAAC	TTCCTCTTGCTTTGATGGG
PCNA	CCACTCTCTTCAACGGTGACACT	TCCCATATCCGCAATTTTATACTCT

GAPDH, glyceraldehyde-3-phosphate dehydrogenase; *KRT10*, keratin 10; *KRT14*, keratin 14; *PCNA*, proliferating cell nuclear antigen; *RPLP0*, ribosomal protein large P0; *TBP*, TATA-binding protein; *TUBA1B*, α-tubulin1B.

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Skin Equivalent and Natural Killer Cells: A New Model for Psoriasis and GVHD

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TO THE EDITOR

The use of human skin grafts in severe combined immune deficiency (SCID) mice has been a powerful research tool for delineating the pathogenesis of psoriasis. The initial model for the induction of psoriasis consisted of human non-lesional psoriatic skin grafted to SCID mice, followed by the injection of human lymphocytes activated with staphylococcal superantigens (Wrone-Smith and Nickoloff, 1996; Nickoloff, 2000a; Boehncke, 2001). This human skin graft/SCID mouse model for psoriasis has also been used to test various therapeutic modalities, such as corticosteroid, cyclosporine and anti-CD11a mAb (Boehncke *et al.*, 1999; Dam *et al.*, 1999; Zeigler *et al.*, 2001). Furthermore, natural killer (NK) cells and T cells with NK receptors (CD94, CD161) can induce psoriasis in human skin grafts on SCID mice (Nickoloff *et al.*, 2000b; Gilhar *et al.*, 2002, 2006).